EXHIBIT 2

The "Normal" Endocrine Cell of the Gut

Changing Concepts and New Evidences

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ABSTRACT: The endocrine cells of the gut are a highly specialized mucosal cell subpopulation. Within the gastrointestinal tract at least 14 different cell types produce a wide range of hormones with a specific regional distribution. The gut endocrine cells belong to the diffuse endocrine system. These cells present two regulated pathways of secretion characterized by large dense core vesicles (LDCV) and synaptic-like microvesicles (SLMV). Gut endocrine cells are recognized by the expression of several "general" markers, including the LDCV marker chromogranin A and the SLMV marker synaptophysin, in addition to the cytosolic markers neuron-specific enolase and protein gene product 9.5. The expression of different hormones identifies specific cell types. The gut endocrine cells are reputed to be terminally differentiated and incapable of proliferation. However, some data suggest that the number of gut endocrine cells may adapt in response to tissue-specific physiological stimuli. Gut endocrine cell differentiation appears to follow a "constitutive" tissue-specific pathway, which may be disrupted and investigated by genetic manipulation in mice. It is suggested that endocrine cell homeostasis is maintained by the entry of new endocrine-committed cells along the differentiation pathway and that such intermediate cells may be sensitive to physiological stimuli as well as transforming agents.

KEYWORDS: gut; endocrine cells; hormones; immunohistochemistry; electron microscopy; transgenic mice; KO

HISTORICAL BACKGROUND

The history of the cells of the so-called diffuse endocrine system (DES) of the gut (also defined as enteroendocrine cells) parallels the early development of histology

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and histochemistry. During the second half of the nineteenth and early twentieth centuries some unusual cells of the gastric and intestinal mucosa attracted the attention of early scientists.[1-4] The staining properties of such cells were attributed to the interaction with chromium salts[4] and were accordingly labeled as enterochromaffin cells.[5] Concurrently in 1902, Bayliss and Starling[6,7] discovered secretin and established the gut as the source of blood-borne agents, "hormones," capable of eliciting physiological effects at a distance. Epithelial "clear cells" (which failed to take up conventional stains) were described by Feyrter[8] in different organs of the human body and included those displaying intrinsic silver-reducing power (chromaffin cells) previously described by Masson.[9] Grouped in the so-called DES, it was suggested that these cells exerted local, "paracrine," action via production and secretion of peptides or amines.[10]

In the 1960s, Pearse[11] identified epithelial cells capable of storing amines and/or taking up amine precursors which are then transformed into amines by intracellular decarboxylation. Comprising the argentaffin,[9] 5-hydroxytryptamine-storing[12] cells of the gastrointestinal tract as well as other cell types, these cells were grouped in the amine precursor uptake and decarboxylation (APUD) system.[13] The unitarian APUD concept led to the proposal for a common neuroectodermal embryological origin for all endocrine cells of the diffuse endocrine system.[13] This hypothesis was shown to be incorrect, as gut endocrine cells subsequently were shown to arise from the gut endoderm.[14]

At this point it is important to recall that in parallel with the development of the DES concept, a nonconventional, slow-growing epithelial tumor was identified and defined as "karzinoide" (carcinoid, i.e., carcinoma-like) by Öberendorfer.[15] The argentaffin properties of some of these tumors were described by Gosset and Masson (1914) and their relationship with the enterochromaffin cells was soon established.[16]

GENERAL CLASSIFICATION

Since the initial observation of Bayliss and Starling, a large number of hormones have been identified in the gut, so that the gastroenteropancreatic tract is now recognized as the largest endocrine organ of the whole human body.[17] The DES system of the gut is remarkably heterogeneous and is composed of as many as 14 highly specialized epithelial cells of endodermal origin.[18] Two regulated pathways of secretion are recognized in gut DES cells and refer to the complex mechanisms controlling the assembly, storage, and release of two different secretory vesicles defined as large dense core vesicles (LDCVs), the usual, well-known electron-dense granule of the endocrine cell, and the synaptic-like microvesicles (SLMVs) of smaller size than the LDCV and very similar to the synaptic vesicle of nerve endings.[19] Besides releasing hormones into the bloodstream to act on distant tissues, the DES cells of the gut constitute a complex regulatory network whose function includes the local fine tuning of secretion, absorption, motility, mucosal cell proliferation, and possibly immune-barrier control. Such activities are exerted by synthesis, storage, and release of peptide hormones and biogenic amines that are, however, specific for each individual cell type. In addition, gut endocrine cells share with neural cells a number of antigens (see below), usually defined as "neuroendocrine markers,"[20] a finding explaining the term "neuroendocrine" frequently used to connote DES cells and derived tumors.

For a general assessment of the neuroendocrine profile in gut mucosa the first methods developed included several silver impregnation techniques for demonstrating the ability of endocrine cells to take up and reduce silver ions in the absence (argentaffinity, Masson-Fontana stain) or in the presence of added reducing agents (argyrophilia, Grimelius stain). The silver-impregnation techniques, although effective and reproducible under appropriate technical conditions, have now been largely replaced with immunohistochemistry for either cytosol markers such as neuron-specific enolase and protein gene product 9.5 or granular markers associated with LDCVs, such as chromogranins and related fragments, or with SLMVs such as synaptophysin.[21–28] Recently, other antigens derived from both LDCVs and SLMVs have been successfully tested in gut endocrine cells and derived neoplasms. The two isoforms of the ATP-dependent vesicular monoamine transporter (VMAT1 and VMAT2)[29] are differentially expressed in LDCVs of gut endocrine cells, with VMAT1 restricted to serotonin-producing enterochromaffin (EC) cells and VMAT2 to histamine-producing enterochromaffin-like (ECL) cells, pancreatic islet cells, and related tumors.[30–33] The neuroendocrine secretory protein 55, an acidic protein of the granin family located in LDCVs, and the synaptic vescicle protein 2 (SV2), a protein expressed in both LDCVs and SLMVs, are effective and specific diagnostic tools for gastroenteropancreatic endocrine tumors.[34,35] Finally, the neural cell adhesion molecules (N-CAM) in their polysialic acid form (N-CAM-PSA) are intensely expressed on the cell surface of endocrine and nerve tissues and derived tumors especially when poorly differentiated.[36–38]

While the aforementioned markers allow the assessment of the endocrine nature of the cells under study and therefore are defined as "general markers," the full identification of their product(s) is obtained by either electron microscopy or immunohistochemistry for specific hormones/amines and therefore defined as "specific markers."[18] Traditionally, ultrastructural analysis allowed separation of the different endocrine granules according to size, shape, and electron density, thus identifying specific cell types (FIG. 1A). The subsequent isolation of peptide hormones, coupled with immunological *in situ* techniques, allowed assignment of each granule/cell type to a specific hormone (FIG. 1B and C).[39,40] So, as a general rule, different granules store different peptide hormones. However, in a specific cell type, the same granule may store more than one peptide hormone and/or amines (see, for instance, the serotonin-producing EC cell or the GLI/PYY-storing L type cell) (FIG. 1D). In addition, cell types producing the same major hormonal content may display different granule morphology in different species (FIG. 2A and B).

DES cells of gut and pancreas in man are listed in TABLE 1 and include several newly discovered peptide hormones. These comprise xenin in gastric inhibitory polypeptide (GIP)-producing cells[41] and, more recently, ghrelin in gastric P/D1 cells.[42] Notably, gastric ghrelin cells display different ultrastructure of the endocrine granules in different species (FIG. 1B).[42–44] As indicated in TABLE 1, although the entire gastrointestinal tract and the pancreas display a well-developed endocrine cell compartment, several cell types are restricted to specific gut sites. As an example, in man, insulin-producing B cells are found exclusively in the pancreas and, along the same lines, histamine-producing ECL cells are restricted to the gastric oxyntic mucosa (TABLE 1). So it appears that tissue-specific differentiation programs control the "normal" endocrine cell maturation in the gastroenteropancreatic tract.

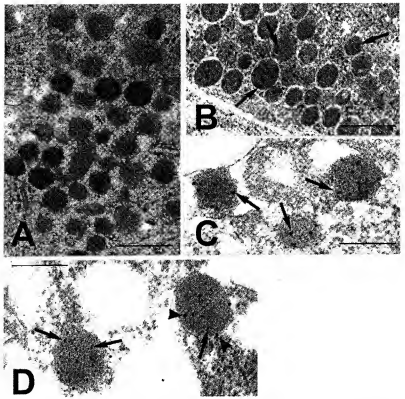

FIGURE 1. Ultrastructural features of L cells in colon tumors of PYY/Tag transgenic mice.[55] The electron-dense, thin-haloed, endocrine granules have a mean diameter 233 ± 47 nm (range 135–399) typical of colon L-type cells (**A**, bar = 600 nm) and display diffuse single immunogold labeling for glicentin (**B**, bar = 400 nm) and PYY (**C**, bar = 250 nm) with 15-nm gold particles (*arrows*). Both antigens are present in the same granules, as demonstrated by double immunogold staining for PYY (*large arrowhead*, 10-nm gold particles) and glicentin (*arrows*, 15-nm gold particles) (**D**, bar = 250 nm); aldehyde-osmium/Epon,Araldite, uranyl-lead citrate.

PROLIFERATION AND DIFFERENTIATION

TABLE 1 provides a static snapshot of endocrine cells in gut and pancreas. Considering their multiple systemic and local roles, DES cells may need to be considered an active cell subpopulation that may adapt and respond to different physiological and pathological stimuli. As will be discussed, endocrine cells in the stomach and intestine may be capable of changing their number and possibly their phenotype in response to stimuli. Although not discussed here, pancreatic endocrine cells may also respond to their environment.

TABLE 1. Type, hormonal content, vesicle markers, and regional distribution of endocrine cells of gut and pancreas in man

	Hormones		Vesicular markers		Stomach			Intestine					
								Small			Large		
Cell type	Peptide	Amine	LDCV	SLMV	Pa	CF	An	D	J	I	Ap	C	R
P/D$_1$	Ghrelin		CgA,VMAT2		e,f	+	f	f	f	f			
EC		5HT	CgA,VMAT1	Syn	f	+	+	+	+	+	+	+	+
D	Som		CgA	Syn	+	+	+	+	+	f	f	f	f
L	GLI/PYY		SgII>CgA	Syn				f	+	+	+	+	+
A	Glucagon		CgA>SgII VMAT2	Syn	+	e							
PP	PP		CgA,SgII, VMAT2	Syn	+			e					
B	Insulin		CgA,VMAT2 NESP5	Syn	+								
ECL		Hista-mine	CgA, VMAT2	Syn		+							
G	Gastrin		CgA	Syn			+	+					
CCK	Cholecys-tokinin							+	+	f			
S	Secretin	5HT	CgA					+	+				
GIP	GIP/Xenin		CgA					+	+	f			
M	Motilin							+	+	f			
N	Neuro-tensin		CgA					f	+	+			

ABBREVIATIONS: LDCV, large dense-core vesicles; SLMV, synaptic-like microvesicles; Pa, pancreas; CF, corpus-fundus; An, antrum; D, duodenum; J, jejunum; I, ileum; Ap, appendix; C, colon; R, rectum; +, presence of cells; f, presence of few cells; e, presence of cells in fetus and newborn; EC, enterochromaffin cell; 5-HT, 5-hydroxytryptamine; Som, somatostatin; GLI, glucagon-like immunoreactants (glicentin, glucagon-37, glucagon-29, GLP-1 and -2); PYY, PP-like peptide with N-terminal tyrosine amide; PP, pancreatic polypeptide; ECL, enterochromaffin-like cell; §, substance P, neurokinins, opioids, guanylin, and other peptides; GIP, gastric inhibitory polypeptide; CgA, chromogranin A; SgII, secretogranin II (also known as chromogranin C); >, heavier staining than; VMAT1,2, vesicular monoamine transporter 1, 2; NESP55, neuroendocrine secretory protein 55; Syn, synaptophysin.

NOTE: This table is adapted from Solcia *et al.*[18] and reflects the current status of knowledge which, especially for the vesicle markers, is largely incomplete.

GASTRIC MUCOSA

The human gastric mucosa displays at least 5 different endocrine cell types (TABLE 1).[18,39,40,45] In the acidopeptic mucosa the histamine-producing ECL cells are the largest fraction, whereas in the antrum, gastrin-producing G cells predominate. Ghrelin-producing P/D1 cells are well represented mainly in the acidopeptic mucosa (up to 10–15% of all endocrine cells), whereas in the antrum, they represent a minor cell population.[42] Somatostatin-producing D cells are found in the entire gastric mucosa, as are the relatively rare serotonin-producing EC cells.

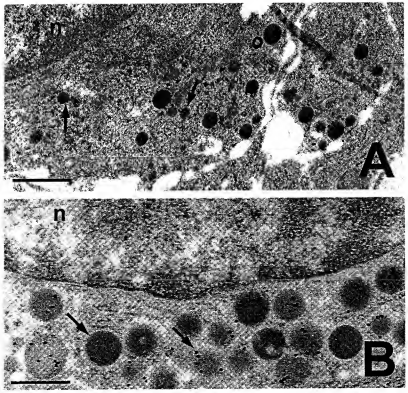

FIGURE 2. Ultrastructural features of gastric ghrelin cells.[40] Immunogold labeling of gastric ghrelin cells in man (**A**, *arrows*, 20-nm gold particles) is observed in electron dense round granules of 130–150 nm in size, typical of P/D1 type; in dog gastric mucosa, ghrelin immunogold labeling (**B**, *arrows*, 20-nm gold particles) is observed in solid granules of ~280 nm in size, typical of dog X cells; aldehyde/LWR, uranyl-lead (**A**, bar = 440 nm); aldehyde-osmium/Epon-Araldite, uranyl-lead (**B**, bar = 416 nm).

In man the "normal" endocrine cells of the gut are reputed as terminally differentiated and nonproliferating as shown by the absence of staining for the proliferation marker Ki67 in chromogranin A-expressing cells.[46] Indeed, in our experience, 5-bromodeoxyuridine (BRDU) incorporation in human gastric mucosa labeled actively replicating cells in the mid crypt zone (the neck area), but not endocrine cromogranin A-positive cells (FIG. 3). Nonetheless, changes in the number of ECL, G, and D cells have all been demonstrated in the human stomach. G cell hyperplasia, reduced D cell counts, and an abnormal G/D cell ratio have been observed in adults and children as due to either impaired acid production, gastritis, and/or idiopathic conditions (see Solcia *et al.*[18] and references therein). ECL, G, and D cells are all involved in the local, physiological control of parietal cell acid production, so that G cells respond to high pH by releasing gastrin, ECL cells in turn respond to gastrin

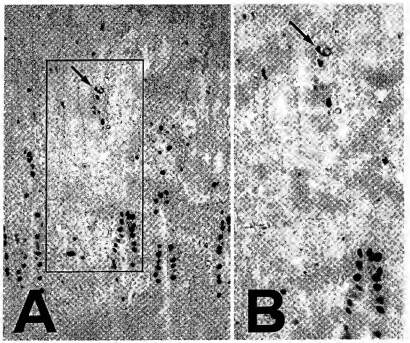

FIGURE 3. Chromogranin A expression in human gastric mucosa exposed to 5-bromodeoxyuridine (BRDU). Strong black nuclear labeling for BRDU is observed in cells of the mid-part of the mucosa (**A**, ×150), where the actively replicating stem cell niche is located. Conversely, intense brown cytoplasm staining for chromogranin A (*arrow*) is found in discrete cells migrated towards the lumen which, however, do not display concurrent BRDU nuclear labeling (**B**, ×180, enlargement of the square in **A**); ABC method, double immunoperoxidase, hematoxylin counterstain.

stimulus by releasing histamine, thus activating the acid-producing parietal cells, whereas D cells participate in both responses in a modulatory local crosstalk.[47] Indeed, gastric luminal hypoacidity, if sustained enough, may result in G cell hyperplasia and reduced D cell numbers of the antrum, whereas the consequent hypergastrinemia determines ECL cell hyperplasia with no significant D cell change of the oxyntic mucosa. Notably ghrelin cells appear not to be under the local acid/gastrin feedback control.[48] All of the foregoing support the view that the stomach mucosa adapts its endocrine cell population to specific physiological and pathological stimuli.[49] Since no direct evidence of endocrine cell proliferation has been demonstrated in man,[46] the changes in endocrine cell numbers should be ascribed to the entry of new endocrine-committed cells along the mucosal differentiation pathway.

The differentiation of gastric endocrine cells appears to be constitutively regulated in a tissue-specific manner, as demonstrated by genetic manipulation in the mouse. CCK-B/gastrin receptor (CCK2R) gene knockout (KO) mice, despite rela-

tively normal reproduction, growth, and development, display abnormal gastric histology, with disrupted ECL cell differentiation.[50–52] CCK2R-deficient mice have increased intragastric pH due to a markedly impaired ability to secrete acid. This defect results from (1) a reduction in the number (30%) of parietal cells, and (2) the relatively inactive status of the parietal cells that are present. High gastric pH is in turn coupled with increased levels of circulating gastrin, increased numbers of antral G cells, and reduced numbers of antral somatostatin D cells. A corresponding increase in the G/D cell ratio is observed. In the oxyntic mucosa, a paradoxically reduced number of histamine-producing ECL cells is found. In addition, the number of somatostatin D and ghrelin cells of the oxyntic mucosa remained unchanged. All these findings indicate that the homeostasis of oxyntic ECL cells requires the expression of CCK2R, pointing to its pivotal role not only for the control of gastric secretion, but also, and more importantly, for the oxyntic-specific differentiation program of endocrine cells. It is also important to note that the absence of CCK2R gene expression does not impair the mucosal response to specific physiological stimuli (namely, luminal hypoacidity) in the antrum, allowing the physiological recruitment of new gastrin G cells and the reduced entry of somatostatin D cells. This finding supports the hypothesis that the differentiation pathway of gastric endocrine cells even in a single organ, the stomach, follows tissue-restricted regulated pathways of differentiation.

INTESTINAL MUCOSA

At least 10 discrete endocrine cell types are found in the human intestinal mucosa with a fairly distinct regional distribution. The serotonin-producing EC cells are the prevalent type and are distributed throughout the entire intestine, as are the somatostatin D cells. By contrast ghrelin, gastrin, CCK, motilin, neurotensin, GIP, and secretin cells are restricted to the small intestine, mostly its upper part, whereas GLI/PYY–producing L cells predominate in the large intestine.

In the small intestine, the variety of hormonal products may suggest tissue-specific paracrine function(s) for the enteroendocrine cells, yet little is known about the local hormonal control of intestinal function(s). In man, much like in the stomach, normal enteroendocrine cells appear to be a terminally differentiated non-proliferating population.[46] Nonetheless, increased numbers of duodenal endocrine cells have been reported for CCK and somatostatin D cells in celiac disease and other rare congenital disorders and for gastrin G cells in the Zollinger-Ellison syndrome.[18] In addition, hyperplasias of unspecified "argyrophil" endocrine cells have been reported in chronic inflammatory bowel disease.[53–55] Except for the regulatory role of proglucagon-derived fragments (with special reference to glucagon-like polypeptide 2) on the intestinal mucosa,[56] relatively little is known about cell number control of enteroendocrine cells.

Genetic manipulation in mice shed some light on the complex differentiation pathway of enteroendocrine cells. Inducible ablation of secretin cells was obtained in transgenic mice expressing herpes simplex virus thymidine kinase (HSV-TK) under the control of 1.8 Kb of 5′ flanking sequence of the rat secretin gene (Sec).[57] HSV-TK expression in transgenic mice is nontoxic. However, the viral enzyme renders proliferating cells sensitive to the nucleoside analog ganciclovir by inhibiting

DNA synthesis and thus allowing conditional cell ablation. In Sec-HSVTK mice, almost the entire population of secretin-producing cells of the small intestine was deleted after 5 days of ganciclovir treatment. In addition, partial ablation of other enteroendocrine types including GIP, 5HT, and somatostatin cells was observed, although without significant change in the number of gastrin G cells. The ablation procedure induced cell death at the neck of the intestinal gland, thus identifying a population of proliferating crypt cells that express secretin at low levels. Overall, these data provided grounds for the proposal of a complex differentiation scheme in which endocrine-committed, multipotent cells are identified. These cells display both proliferative capacity, since they are sensitive to a transgenic construct with an inducible toxic phenotype, and multiple differentiation capacity, since their ablation results in the depletion of different endocrine cell types. Incidentally, these observations indicate that the endocrine commitment takes place in cells close to the mucosal stem cell niche, as known from classic H3-thymidine labeling, autoradiography, and electron microscopy data.[58]

In the large intestine, targeting GLI/PYY-producing L cells with an hybrid oncogene construct determined the development of colonic well-differentiated endocrine tumors.[59] The endocrine tumors were composed either of the GLI/PYY-producing L cells (FIG. 1), as expected from the transgene construct, but also of several other enteroendocrine cell types. The search for coexpression of PYY and other hormones in colon endocrine cells of normal and transgenic mice during development and adult life demonstrated that PYY is expressed early during development, it accompanies the expression onset of other gut hormones, and its coexpression persists after birth in discrete endocrine cells. These observations suggest that PYY is expressed in an endocrine-committed multipotent cell and that PYY expression is progressively lost during subsequent differentiation. This endocrine-committed PYY-expressing cell displays discrete multiple endocrine differentiation capacity and appears to be sensitive to transforming agents.

CONCLUDING REMARKS

The foregoing evidence in the stomach and the intestine supports the view of gut endocrine cells as a mucosal population actively adapting to local stimuli. The "normal" endocrine cell equilibrium appears to be maintained by tissue-restricted, "constitutive" differentiation programs thst require multiple tissue-specific effectors. Changes in endocrine cell numbers are probably mediated by the entry of new cells along the differentiation path of the mucosa and by specific physiological demands. The differentiation trail of endocrine cells is characterized by multiple hormone gene expression which follows complex tissue-specific hierarchies during development and adulthood. Endocrine-committed intermediate cells are possibly the proliferating elements responsive to tissue-specific physiological stimuli. It is suggested that these intermediate cells, located close to the totipotent stem cell niche, are also sensitive to transforming agents and may generate endocrine tumors. The recent analysis of gene expression of enriched gastric and intestinal stem cells[60,61] may hopefully help in understanding the molecular basis of endocrine cell restriction in the gut.

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